

BIOTECHNOLOGICAL CONDITIONS OF VALVE PROSTHESES CREATING BY TISSUE ENGINEERING METHOD

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Nowadays, definitive treatment for the end-stage organ failure is transplantation. Tissue engineering is an up to date solution to create the effective substitute of the defective organ. It involves the reconstitution of viable tissue with the use of autologous cells grown on connective tissue matrix, which has been acellularized before. Basis for the prosthesis should be morphologically and physically nonmodified, so in case of making vessel-valvular biological prostheses the decellularized extracellular matrix is the best variant. The xenogeneic extracellular matrix is economically and ethically more useful. The possibility of preservation of the morphological and chemical properties of matrix structure initiates the process of programmed cell death. In contrast to necrosis, which is a form of traumatic cell death that results from acute cellular injury, apoptosis doesn't cause the tissue damages. One of the ways of realizing the apoptosis is the usage of EDTA — chelate, which binds the Ca²⁺ ions.

Key words: tissue engineering, extracellular matrix, decellularization, apoptosis, chelate.

Tissue engineering is the advanced biotechnology area focusing on invention of artificial substitutes of biological origin to repair, support and activity improvement of the defective organ or its part [1, 2]. In this case the therapeutic reconstruction is achieved by delivering support structures (matrix), cells as well as regenerative molecular and mechanical signals to the specific area [3, 4].

Today the tissue engineering covers a range of materials studied as matrices [1, 5]. Nominally they can be divided into two categories: polymer matrix, including biological and synthetic polymers, and connective tissue frameworks of human (allogeneic) and animal (xenogeneic) tissues [6, 7].

According to one of the most inspiring hypotheses in cardiological surgery the tissue engineering is an opportunity to create a perfect valvular prosthesis — live, growable, adaptive, autologous and functionally optimally adjustable [8, 9]. The point of this hypothesis is to use the valvular connective tissue frameworks, which after decellularization [10, 11] become perfect for prosthesis repopulation with recipient cells and creation of a live autologous tissue [12, 13].

Using allogeneic decellularized grafts is limited by insufficient amount of transplants. That is why xenogeneic tissue becomes increasingly popular as a basis for manufacturing tissue-engineered valvular prostheses [14, 15]. This biotechnological approach will make valvular prosthetic repair procedure more widely applicable due to material availability [6, 16, 17].

It is especially convenient to use porcine valves regarding their 3D structure and extracellular matrix structure, which are much similar to the relevant parameters of the human valve [17]. It is also important that molecules of the extracellular matrix refer to the class of highly conserved proteins (their amino acid composition has low variability among different species), that almost completely explains the absence of immune response after decellularized tissue xenografting [18, 19]. Additionally it is presumed that except immune response decrease the decellularization will contribute to life improvement of valvular transplants [20–22].

Decellularization is an intentional initiation of cell death in the biological tissue [23–25]. It is significant that there are two basic ways of tissue and human/animal

organ cell death — apoptosis and necrosis. Respectively all known contemporary methods of biotechnological practice for decellularization can be divided into two basic groups: necrosis incurring cell death methods and methods inducing cell apoptosis [24].

Necrosis-induced decellularization methods have their advantages and disadvantages. For example, such widely applicable operations as enzymatic processing, hypotonic shock and treatment with SDS (sodium dodecyl sulfate) [26] allow to achieve a highly efficient decellularization for a short time. However, at the same time, tissues processed in such a way get destructed after transplantation because of tissue calcification.

The reason is that necrosis generally begins with damaging plasma membrane and as a result cells lose their ability to preserve ionic homeostasis. As a result of plasma membrane ruptures the cytoplasm content, including lysosomal enzymes, gets into extracellular space. It leads to significant tissue damages with active inflammatory process [27]. It is assumed that tissue with cells which died due to necrosis calcifies faster, because dead cells are an obligatory condition for local concentration change of calcium, phosphates, proteins, lipids, enzymes providing deposition of soluble calcium phosphates and at some conditions their transition to the insoluble state [28].

In contrast to necrosis the apoptosis-induced cell death takes place under physiological conditions. Apoptosis is a programmed cell death, energetically dependant, genetically controlled process, which under *in vivo* conditions is launched by specific signals and recovers the organism from weak, unwanted and damaged cells [27] according to the morphogenesis and organism individual development program. Such cell death type involves an activation of non-lysosomal endogenic nucleases splitting nuclear DNA into small fragments. Apoptosis important feature, making it different from necrosis, is absence of inflammable reaction of neighboring cells for decay products because degrading cell keeps membrane unbroken till final process stages and then undergoes phagocytosis by microphages or surrounding cells. Characteristic features of apoptosis generally include: dehydration cell shrinkage; intercellular junction loss; blebbing — appearance of blisters on cell membrane; cytoskeleton destruction; chromatin condensation; nuclear fragmentation and DNA degradation [29].

Three morphologically separated stages are specified in apoptosis process: signal (induction) stage, effector stage and degradation (destruction) stage. Apoptosis inductors may be external (extracellular) factors as well as intracellular signals [27]. The signal is accepted by the receptor and then transferred sequentially to the mediator molecules (messengers) of various order and gets to the nucleus where cell “suicide” program is engaged through lethal gene activation or non-lethal gene repression. The nucleus experience first apoptosis morphological characteristics: chromatin condensation and formation of osmiophilic patches at nuclear membrane. Later membrane invaginations (excavations) appear and the nucleus is fragmented. The basis for the chromatin degradation is enzymatic DNA splitting [8, 19]. Getting past this stage makes the process non-reversible. Then internucleosomal DNA disintegration starts, i.e. DNA strings between nucleosomes are destroyed. Departed nucleus parts, covered with membrane are called apoptotic bodies. The cytoplasm endures endoplasmic reticulum broadening, condensation and granule retraction. Cell membrane loses villosity and gets blisters. Cells are rounded up and separate from substrate. Different phagocyte identified molecules emerge on the surface: phosphoserine, trombospondin, desialylated membrane glycoconjugates, after what the cell body in apoptosis is absorbed *in vivo* by other cells (macrophages and neighboring cells) and retrogrades surrounded by lysosome of phagocytic cells [8, 27], besides, as it was mentioned before, this process is immunologically passive and is not accompanied by inflammation reaction or tissue destruction [8].

Methodological approaches for apoptotic death identification are usually divided into several groups according to considered criteria of a given apoptosis stage. These include: 1) cell size decreasing and density rise; 2) composition change in cell external membrane; 3) selective fragmentation of nuclear DNA; 4) content change of apoptosis molecular markers (or their redistribution in a dying cell) [30]. In this way according to the listed criteria the first group of methods can be singled out including routine light microscopic study, using conventional fixing and painting methods or means of selective discovering of pyknotized chromatin. These also include methods using vital stain. The second group includes fluorescent microscopic study, using

fluorochromes and flow cytophotometry. The third group of apoptosis verification methods includes electron microscopy techniques. The fourth method identifies apoptotic changes in cells through in situ identifying oligonucleosomic DNA degradation. The fifth group uses immunohistochemical identification of protein markers taking part in apoptosis activation [30].

It is significant that proving the fact of apoptotic cell death on the results of morphological identification methods only which use light microscope is one of the most common ways, but still lacks reliability. It is regarded [31] that morphological changes during apoptosis become visible under light microscopic study only after non-reversible changes in the nucleus and cytoplasm. The changes at early apoptosis stages can be identified only under additional laboratory procedures.

Moreover morphological apoptosis verification criteria are rather controversial. Many authors find it ambiguous that such morphological signs as karyopyknosis, karyorrhexis or cytoplasmic staining are apoptosis specific [32].

For example, nuclear pyknosis is connected with specific internucleosome chromatine degradation. Although such changes can be witnessed during large scale molecular DNA cutting at early apoptosis stages, it is still not exclusive for apoptotic cell death. Pyknosis cannot be called an absolute apoptosis verification sign because of the reason that nuclear pyknosis patterns are witnessed under large number of pathologic processes accompanied by dystrophic cell changes [30].

Cytoplasmic staining (basophily at early apoptosis stages) is at one point connected with maintaining protein synthesis during apoptosis and at other point with transglutaminase activity rise resulting in cytoplasm thickening [33]. Such biochemical features distinguish the apoptosis from necrosis, however, their morphological expression is nor exclusively characteristic for apoptotic cell death as it can be connected with cell's metabolism state, and this enables using morphological changes as an additional apoptosis criterion.

For more precise apoptotic cell death identification the methods of the mentioned fourth study group are often used. These include, for example, a specific apoptosis stain under Apoptag® Peroxidase ISOL Kit (Chemicon) system based on fragments DNA detection by TUNEL-method (Terminal deoxynucleotid Transferase — mediated

dUTP — biotin Nick — End Labeling) through enzymatic staining of free 3'-OH ends of single- or two-stranded DNA breaks with acting deoxynucleotid transferase enzym [34]. In some cases this method relates to immunohistochemical studies [33]. That is wrong because such analysis does consider the interactions between antigen and antibody.

As for apoptosis activation methods it is usual to include hypothermic tissue processing as well as using calcium free solution ethylenediaminetetraacetic acid (EDTA) [34].

EDTA is a chelate capable to bind calcium ions so that cadherin interaction is disturbed and the physical cell connection into a single tissue decreases. This brings to cell dissociation [33, 35]. At the same time higher EDTA concentrations, according to the literature data, can activate apoptosis process [35]. In addition EDTA as calcium, magnesium and other metal ions chelator is used to restrain accumulation of calcium and phosphates in mitochondrias during cell death. Nevertheless it is still remarkable that according to many studies [35, 36] this decellularization method does not provide a complete removal of dead cells from matrix stratum, what is obviously connected with cell ability to migrate from the surface of the processed tissue into its stratum due to a negative chemotaxis. However, such decellularization method promotes apoptotic death of donor's cells in transplants enabling full realization of the process under in vivo conditions and implication of, for example, macrophages, which, as it was mentioned, are responsible for in vivo elimination of apoptotic cells.

It should be noted that except elimination of donor's tissue cells the successful obtainment of autologous vital valvular transplant depends on 3 elements: appropriate autologous cells (by phenotype and functions); matrix as a temporary carrier providing tissue durability until extracellular matrix is synthesized by autologous cells; and opportunity of tissue growth and complete development under in vitro conditions which are close to the physiological [26, 36].

Durability increase, successful implant survival [23], transplant self-renewal and thrombogenesis risk decrease [12] directly depend on preserving matrix sustainability decellularized with extracellular basis as well as on penetration velocity of tissue-forming cells of a recipient organism, their population and matrix formation rate [35].

Extracellular matrix is considered according to its role in supporting structure

and 3D-form of a particular organ and its basis. It is a so called dynamic connection to the resident cell population [22, 37, 38] whose phenotype including genetic profile, protein composition and functionality depends on microenvironment conditions (holes), with such factors as oxygen concentration, pH, mechanical forces and environment biochemistry [39].

Matrix scaffold produced by decellularization can support and stimulate appropriate cell phenotype in repopulation process by ligands and bioactive molecules, necessary for resident and migrating cells for their self-organization into functional groups and forming a stabile structure and functionality [39–41].

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БИОТЕХНОЛОГІЧНІ УМОВИ СТВОРЕННЯ ПРОТЕЗІВ КЛАПАНІВ МЕТОДОМ ТКАНИННОЇ ІНЖЕНЕРІЇ

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У наші дні трансплантація на термінальних стадіях захворювань, пов'язаних із ушкодженням органа, часто є єдиним можливим методом лікування. Тканинна інженерія — це сучасне рішення, що дає змогу сконструювати ефективний замінник ушкодженого органа. Продукт тканинної інженерії дозволяє відновити життєздатність тканини, його основою є попередньо децелюльований сполучнотканинний матрикс з аутологічними клітинами на ньому. Такий матрикс має бути морфологічно і фізично незмінним. З економічного та етичного погляду перевага належить ксеногенному екстрацелюлярному матриксу. Можливістю збереження морфологічних і хімічних властивостей матричних структур є ініціювання процесу програмованої загибелі клітин. На противагу некрозу, що призводить до травматичної загибелі клітин, яка відбувається внаслідок гострого клітинного ушкодження, апоптоз не є причиною ушкодження тканини. Один із шляхів реалізації апоптозу — використання ЕДТА — хелату, що зв'язує іони Ca^{2+} .

Ключові слова: тканинна інженерія, екстрацелюлярний матрикс, децелюляризація, апоптоз, хелат.

БИОТЕХНОЛОГИЧЕСКИЕ УСЛОВИЯ СОЗДАНИЯ КЛАПАННЫХ ПРОТЕЗОВ МЕТОДОМ ТКАНЕВОЙ ИНЖЕНЕРИИ

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В наши дни трансплантация на терминальных стадиях заболеваний, связанных с повреждением органа, часто является единственным возможным методом лечения. Тканевая инженерия — это современное решение, позволяющее сконструировать эффективный заменитель поврежденного органа. Продукт тканевой инженерии позволяет восстановить жизнеспособность ткани и представляет собой основу из предварительно децелюлированного соединительнотканного матрикса с аутологичными клетками на нем. Такой матрикс должен быть морфологически и физически неизменным. С экономической и этической точки зрения преимущество принадлежит ксеногенному экстрацелюлярному матриксу. Возможностью сохранения морфологических и химических свойств матричных структур является иницирование процесса программируемой гибели клеток. В противоположность некрозу, приводящему к травматической гибели клеток, которая происходит вследствие острого клеточного повреждения, апоптоз не является причиной повреждения ткани. Один из путей реализации апоптоза — использование ЭДТА — хелата, связывающего ионы Ca^{2+} .

Ключевые слова: тканевая инженерия, экстрацелюлярный матрикс, децелюляризация, апоптоз, хелат.